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ON THE PLACENTAL TRANSFER OF VITAMIN C

An experimental study on guinea pigs and human subjects

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VAMMALA 1958

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FROM THE INSTITUE OF OCCUPATIONAL HEALTH, HELSINKI,
THE CHILDREN'S CLINIC OF THE UNIVERSITY OF HELSINKI,
THE RESEARCH LABORATORIES OF THE STATE ALCOHOL MONOPOLY, HELSINKI
AND FROM THE WENNER-GREN CARDIOVASCULAR RESEARCH LABORATORY,
STOCKHOLM.

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Contents.

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Helsinki, October 1958.

Niels Räihä

I. Introduction

»Just over 200 years ago, James Lind summarized his medical experience in the British navy in his treatise on 'the scurvy'. He showed its cause, described its cure, demonstrated its prevention, and defined its treatment. The Lords of the Admirality adopted Lind's recommendations officially for the Royal Navy after a lapse of 40 years. But 125 years were required before students of chemistry and physiology finally isolated and synthesized ascorbic acid and demonstrated its functions in relationship to glutathione, codehydrogenase II, sulfhydryl enzymes, phosphatase, carbohydrate metabolism, and the intermediary action of folic acid and its conversion into folinic acid» (*Bean* 1957, p. 173).

Studies on maternal nutrition during pregnancy have shown the important role of vitamins in the maintenance of a normal gestation and fetal development (*Utheim-Toverud* 1939, *Burke* et al. 1943 and *Utheim-Toverud, Stearns* and *Macy* 1950). It is also well known that the susceptibility of the female to scurvy is greatly increased during pregnancy.

The high concentration of vitamin C in the fetal blood (*Wahren* and *Rundqvist* 1937, *Neuweiler* 1938, *Braestrup* 1938, *Teel, Burke* and *Draper* 1938, *Manahan* and *Eastman* 1938, *Elmby* and *Becker-Christensen* 1938, *Braestrup* 1939, *Snelling* and *Jackson* 1939, *Möller-Christensen* and *Thorup* 1940, *Mindlin* 1940, *McDevitt* et al. 1942, *Lund* and *Kimble* 1943, *Slobody, Benson* and *Mestern* 1946 and *Hamil* et al. 1947), the high concentration in the first urine passed (*Hamil* et al. 1947) and the subsequent decrease in the tissue saturation after birth (*Ingalls* 1938) suggest that the physiological processes of intrauterine

life require greater tissue saturation of vitamin C than is necessary during extrauterine existence.

Ingier (1915) produced scurvy in the fetuses of guinea pigs on the 10th to 15th day after the mothers diet had been changed; the litters were frequently premature or stillborn, and showed retarded growth. These results have later been confirmed by *Kramer, Harman* and *Brill* (1933). In an unpublished experimental series in which the author kept 10 pregnant guinea pigs on a vitamin C free diet at the end of gestation, the litters were stillborn in 7 cases. *Sauvage Nolting* (1955) reports a defective brain development in human subjects caused by vitamin C deficiency.

In a material consisting of 2,129 pregnant women, *Martin* et al. (1957) found an increased frequency of premature birth in those mothers with the lowest intake level and the lowest serum concentration of vitamin C. *Pankamaa* and *Räihä* (1957) have shown a close negative correlation between the seasonal fluctuation of stillbirths and that of vitamin C in fetal tissues. In a material comprising 116,790 deliveries at the Women's Clinic of the University of Helsinki the lowest percentual frequency of stillbirths occurred in September and the highest in January. According to the State Bureau of Statistics the total number of births in Finland during the year 1956 was 90,553, of which 1,657 were stillbirths. The frequency of stillbirths was highest, (2.03 %) in December and January, and was lowest (1.50 %) in September.

II. Plan of study

The problem of the mechanism of transfer of vitamin C across the placental barrier from the maternal to the fetal circulation is far from settled and only a few suggestions based on indirect experimental data have been presented in the literature.

The investigations to be described were undertaken with the purpose of elucidating the process of transfer of vitamin

C from mother to fetus, since the vitamin seems to be of great importance for a normal gestation and fetal development.

The following plan of study developed during the course of the experimental work:

1. To study the methods available for the determination of L-ascorbic acid, dehydroascorbic acid and total ascorbic acid in plasma and whole blood, and to find a method suitable for the estimation of these substances in small samples of blood.

2. To study and compare the permeability of adult and fetal erythrocytes from human subjects and from guinea pigs to L-ascorbic acid and dehydroascorbic acid, and to throw some light on the mechanism of transfer of ascorbic acid across the erythrocyte cell membrane.

3. To study the transfer of L-ascorbic acid and dehydroascorbic acid across the human and the guinea pig placenta from mother to fetus.

III. Material and Methods

1. Experimental animals

Guinea pigs were chosen as the experimental animal for the following reasons: 1) the guinea pig cannot synthesize vitamin C, and scurvy develops rapidly when the vitamin is excluded from the diet, as shown already in 1907 by *Holst* and *Frölich;* 2) the guinea pig placenta has many morphological features in common with the human placenta, and they are both haemochorial according to the classification of *Grosser* (1909, 1927); 3) at the end of gestation, the fetuses of guinea pig are sufficiently large to make it easy to collect blood samples from the umbilical vessels.

The guinea pigs used were obtained from the colony of the Institute of Occupational Health in Helsinki, and from the animal stables of Orion Pharmaceutical Mfrs. in Helsinki. The exact age of the fetuses was not known.

2. Human material

The human material comprises 9 cases of legal abortion performed at Södra Barnbördshuset, Stockholm, and samples of maternal blood, cord blood and amniotic fluid from 41 normal deliveries at the Women's Clinic of the University of Helsinki. In ten of these 41 cases, the mothers stayed at the hospital some days prior to the expected delivery. Two g of L-ascorbic acid per day was administered *per os* to these mothers until delivery.

The amniotic fluids were collected by puncture prior to delivery, or at spontaneous rupture of the membranes.

The blood samples from the mothers were drawn during delivery.

The samples of cord blood were obtained from the umbilical vein at delivery.

The blood samples were heparinized, and stored in a refrigerator until the analysis was carried out. All the samples were assayed within some hours after collection. No loss of ascorbic acid from plasma or whole blood was observed during this time.

3. Experimental technique

The experimental technique used in the animal experiments was mainly the same as described by *Karvonen* and *Räihä* (1954), except for a few modifications and additions.

The animals were given a light intraperitoneal »Nembutal» anesthesia, 3 mg per 100 g. Both the maternal carotid arteries were cannulated with polyethylene tubing, and an initial maternal blood sample was drawn. The fetuses were delivered by means of cesarean section into a 0.9 % sodium chloride bath kept at + 37°C. In the experiments in which the effect of hypothermia was studied, one of the fetuses was delivered into a well insulated container kept at about + 27°C, while the rest of the fetuses were kept in the original bath at + 37°C. The cold container was placed in such a way that it could hold the entire fetus and the placenta without seriously obstructing the umbilical vessels.

Initial samples were drawn from the amniotic sac and from the umbilical vein of the fetuses.

Two kinds of experiment were carried out. In one kind, physiological saline solutions of L-ascorbic acid were infused into the maternal circulation by the use of a constant speed infusion pump. In the other form of experiment, dehydroascorbic acid solutions were infused. The concentration of the solutions varied from 20 to 200 mg %, and the total volume infused was 10 ml. The speed of infusion was either 0.5 ml or 1.0 ml per minute. Successive blood samples were taken from the mother and the fetuses during and after the infusion.

In the experiments on the human subjects, the umbilical cord was cautiously exposed by a vaginal hysterotomy made on the mother under ether anesthesia. In three cases, A.F., B.R. and K.K., initial blood samples were drawn from the umbilical vein and from the left antecubital vein of the mother, and immediately an injection of 1.0, 1.0 and 1.5 g respectively of L-ascorbic acid was made into the right antecubital vein of the mother. Maternal and fetal blood samples were collected after 3, 6 and 15 minutes respectively. In experiments B.S., D.V., F.Y. and S.Ö., the L-ascorbic solution was injected into the maternal circulation some time before the exposure and blood sampling of the umbilical cord, as seen in table V (page 36).

Similar experiments on the permeability of the human placenta to glucose, fructose and xylose have recently been reported by *Holmberg* et al. (1956).

4. Analytical

The following preparations were used in the experiments: *L-ascorbic acid* (AA) was supplied by F. Hoffman-La Roche & Co., Basel. *Dehydro-L-ascorbic acid* (DHA) was prepared according to the method of *Pecherer* (1951).

In the experiments on human subjects, *C-vitamin forte* ampules of L-ascorbic acid supplied by Astra, Södertälje were used.

Numerous chemical methods have been suggested for the

determination of vitamin C in biological material. The majority of workers who have investigated the vitamin C content of body fluids have used methods which are based on either the reduction of 2,6-dichlorophenolindophenol for the measurement of AA (*Tillmans* 1928), or the coupling reaction of 2,4-dinitrophenylhydrazine with DHA for the measurement of total ascorbic acid (TAA), i.e. L-ascorbic acid, dehydroascorbic acid and 2,3-diketogulonic acid (*Roe* and *Kuether* 1943). Determinations before and after the reduction with hydrogen sulfide (*Bessey* 1938, *van Eekelen* and *Emmerie* 1936 and *Roe* et al. 1948) and with homocysteine (*Hughes* 1956), or oxidation with activated charcoal, bromine or other oxidizing agents, have been used for the selective determination of AA and DHA. Comprehensive reviews of these methods have recently been published (*Olliver* 1954 and *Roe* 1954).

Estimation of total ascorbic acid in plasma, whole blood and amniotic fluid

When working with fetal blood samples, it was desirable to have an analytical method by which accurate determinations could be made requiring a minimum of blood or plasma, due to the difficulty of obtaining large blood samples from the fetal vessels. In order to establish a specific micro-method for the determination of TAA, the methods of *Lowry, Lopez* and *Bessey* (1945) and that of *Schaffert* and *Kingsley* (1955) were combined.

Principle. — AA is oxidized to DHA by a mild oxidizing agent. When treated with 2,4-dinitrophenylhydrazine, the DHA forms an orange-red acid soluble precipitate, a bis-2,4-dinitrophenylhydrazone, in which the 2,4-dinotrophenylhydrazine is coupled to carbon atoms 2 and 3. When the precipitate is treated with strong sulfuric acid, it undergoes a molecular rearrangement, and a highly stable reddish product is formed which can be measured photometrically.

Procedure. — To a 0.05 ml sample of plasma, whole blood or amniotic fluid 0.2 ml of an acid activated charcoal suspension was added. This suspension was made by placing 1 % acid washed Norit in a 5 % trichloracetic acid solution. The mixture

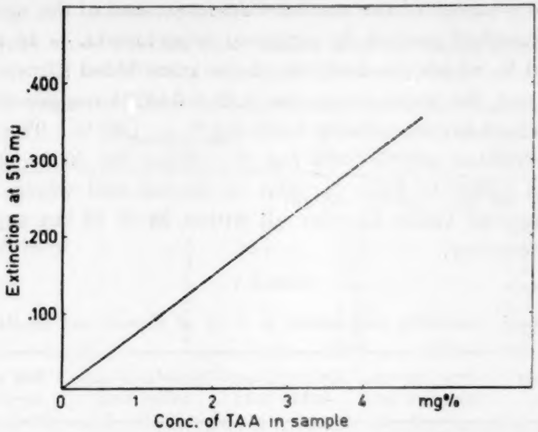


Figure 1. **Relation between the total ascorbic acid concentration in the sample and the extinction at 515 mμ. The curve represents the standardization of the method for the determination of total ascorbic acid.**

was left to stand in a small test tube for 5 minutes, and then centrifuged for 10 minutes at 3000 r.p.m. 0.15 ml of the clear supernatant was transferred to another test tube, and 0.05 ml of a thiourea-2,4-dinitrophenylhydrazine reagent added to the mixture. This reagent was prepared by dissolving 2,4-dinitrophenylhydrazine to the extent of 2 %, and thiourea to the extent of 0.25 %, in 9 N sulfuric acid. The test tube containing the sample and the reagent was placed in a boiling water bath for exactly 5 minutes. At the end of this time, the tube was placed in an ice bath, and 0.25 ml of an ice cold 85 % sulfuric acid solution was added drop by drop, mixed thoroughly, and left to stand for 10 minutes. The color was read in a Beckman spectrophotometer at 515 mμ against a blank prepared in the same manner.

***Standardization.* — A 100 mg % solution of AA in 5 % trichloracetic acid, prepared just before use, was diluted into solutions of 1, 2 and 4 mg%. 0.05 ml of each solution was added to the Norit-trichloracetic acid suspension, and the procedure was continued as above. Figure 1 shows the relation between the concentration of TAA and the extinction at 515 mμ.**

Determination of the standard deviation and of the accuracy of the modified method by recovery experiments. — In an experiment in which ten analyses of the same blood filtrate were carried out, the mean value was 1.28 ± 0.00775 mg per 100 ml, the standard deviation being 0.025 mg % = 1.95 %. The maximum deviation was — 0.05 mg %. When 10, 20 or 40 γ of AA was added to fresh samples of plasma and whole blood, the recoveries (table I) were all within 98 % of the expected 100 % recovery.

TABLE I

Recovery of L-ascorbic acid added to 1 ml of plasma and whole blood.

Sample No.	Original total ascorbic acid	Added L-ascorbic acid	Recovered ascorbic acid	Per cent recovery
Plasma	γ	γ	γ	
1	9.5	10	10.0	100
2	9.5	20	19.9	99
3	9.5	40	40.0	100
Whole blood				
1	13.0	10	9.8	98
2	13.0	20	20.0	100
3	13.0	40	39.3	98

Comparison of the modified micro-method with the macro-method of Schaffert and Kingsley. — The modified method was compared with the original method of *Schaffert* and *Kingsley* (1955) for the estimation of TAA. Table II shows the comparisons of samples of whole blood obtained from 7 normal adults.

Discussion. — The specificity of the 2,4-dinitrophenylhydrazine method for the determination of TAA has been the subject of much discussion and the method is generally considered to have a high degree of specificity. Such interfering substances as D-glucoascorbic acid, D-araboascorbic acid, 2,3-diketogulonic acid, reductic acid and purified reductone have been reported by *Penny* and *Zilva* (1945). Of these, only 2,3-

TABLE II

Comparison of the modified micro method with the method of Schaffert and Kingsley on samples of whole blood.

	Sample No.	Modified micro method	Schaffert and Kingsley
		mg per 100 ml	mg per 100 ml
Whole blood	1	1.30	1.35
	2	0.95	1.10
	3	1.40	1.45
	4	0.85	0.83
	5	1.00	1.05
	6	0.87	0.85
	7	0.68	0.67

diketogulonic acid, which is an anti-scorbutically inactive oxidation product of AA, is of interest when animal samples are analyzed. The other substances mentioned mainly appear in food preparations.

Schaffert and *Kingsley* (1955) state that at lowered pH and at temperatures between 25°C and 50°C, the 2,3-diketogulonic acid develops more color with 2,4-dinitrophenylhydrazine than does an equivalent amount of DHA, and as the temperature increases to 75°C the reaction changes in favor of DHA. At 100°C, there is no significant interference by 2,3-diketogulonic acid. According to *Darmon, Monier* and *Roe* (1952), and *Iggo, Owen* and *Stewart* (1956), only trace amounts of diketogulonic acid are present in blood under normal conditions.

The rate of coupling of sugars with 2,4-dinitrophenylhydrazine is very slow in comparison with that of the oxidation products of AA. The osazones of the sugars also decompose as a result of the high concentration of sulfuric acid used (*Roe* 1954).

The differentiation between L-ascorbic acid and dehydroascorbic acid in plasma

By omitting the treatment of the substrate with activated charcoal, the AA present is not oxidized, and only the DHA is

measured. The content of AA of the sample is obtained from the difference between the TAA and the DHA (*Schaffert* and *Kingsley* 1955). The possibility of the existence of DHA in plasma has been ignored by some authors (*Farmer* and *Abt* 1936, *Kellie* and *Zilva* 1936 and *Borsook* et al. 1937). These authors claim that vitamin C exists in plasma in the reduced form. *Darmon, Monier* and *Roe* (1952), *Barany* and *Langham* (1955) and *Iggo, Owen* and *Stewart* (1956) report only very small amounts or no DHA at all as being present in the plasma of normal humans and guinea pigs. Contrary to these reports, however, *Chen* and *Schuck* (1950) and *Stewart, Horn* and *Robson* (1953) claim that a significant part of the total vitamin C in normal human plasma exists as DHA. Using the 2,4-dinitrophenylhydrazine method before and after oxidation with activated charcoal in order to differentiate between AA and DHA, *Schaffert* and *Kingsley* (1955) found large amounts of DHA in the whole blood and plasma of normal human subjects. *Piha* (1956), using the method of *Roe* and *Kuether* (1943), which is based on the same principle, similarly found large amounts of DHA in the plasma and whole blood of rabbits. As the reports on the existence of DHA in blood and plasma are confusing and contradictory, it seemed necessary to study the extent to which the methods used might have influenced the results.

A standard solution of AA was prepared. DHA and TAA were estimated before and after oxidation with activated charcoal by using the 2,4-dinitrophenylhydrazine method of *Schaffert* and *Kingsley* (1955). The value obtained for TAA agreed to within 98 % with the concentration of the standard solution prepared. The value for DHA, however, was as high as 60 % of the TAA value. This result could be explained only by one of the following assumptions: either a considerable oxidation had occurred in the original dry sample of AA, or some of the AA was oxidized during the determination. In order to solve this problem, the standard solution was tested for AA by the photometric indophenol method of *Mindlin* and *Butler* (1937—1938), before and after a reduction with hydrogen sulfide. The values obtained before and after the treatment with hydrogen sulfide were equal, and agreed to within 99 % with the original

concentration of the standard solution. This test showed conclusively that considerable oxidation of the AA occurs during the determination of DHA by the 2,4-dinitrophenylhydrazine method. This has also been observed by *Darmon, Monier* and *Roe* (1952), and by *Iggo, Owen* and *Stewart* (1956).

Akerfeldt (1957) has suggested a simple method, which is suitable for clinical use, to determine the approximate concentration of AA in plasma. This method is based on the ability of AA to reduce N,N-dimethyl-p-phenylenediamine (DPP) which has been oxidized by the plasma ceruloplasmin.

In the present investigation, the micro method of *Mindlin* and *Butler* (1937—1938) was used for the estimation of AA in plasma and amniotic fluid. The reaction was carried out at a pH between 3.5 and 4.0, at which pH the indophenol is stable (*Roe* 1954, and *Owen* and *Iggo* 1956). The DHA values were calculated as the difference between the TAA and the AA.

The differentiation between L-ascorbic acid and dehydroascorbic acid in whole blood and erythrocytes

In animal tissues in general, the concentration of AA is greater than that of DHA, and it is therefore reasonable to assume that the systems which oxidize and reduce vitamin C under normal conditions are maintained in a balance which tends to keep the vitamin within the cell in the reduced state. When AA or DHA are analyzed in whole blood or in erythrocytes, the physiological balance within the cell is damaged, and the original ratio between reduced and oxidized ascorbic acid in the cell may be altered (*Mapson* 1954, p. 228).

No specific enzyme similar to ascorbic acid oxidase in plants for direct oxidation of AA has been found in animal tissues. Non-enzymatic catalysts such as copper and iron ions are known easily to catalyze the oxidation of AA (*von Euler, Myrbäck* and *Larsson* 1933, *Barron, de Meio* and *Klemperer* 1936 and *Mapson* 1945). *Stotz* et al. (1937—1938), report that the cytochrome c-cytochrome oxidase system has been demonstrated to function as a catalyst for the aerobic oxidation of AA, and that this system is chiefly responsible for the slow oxidation of vitamin C

in excised tissues. Cytochrome c and liver mitochondria constitute a known system for the oxidation of AA to DHA (*Maley* and *Lardy* 1954 and *Lehninger, Ul Hassan* and *Sudduth* 1954).

The systems responsible for the reduction of DHA have not as yet been conclusively demonstrated in animal tissues. The action of sulfhydryl (—SH) groups, especially of glutathione as a hydrogen donor in the reduction of DHA, has been suggested by several workers (*Szent-Györgyi* 1928, *Borsook* et al. 1937, *Schultze, Stotz* and *King* 1937—1938 and *Hopkins* and *Morgan* 1943). *Christine* et al. (1956) suggest that a still unknown enzyme, similar to the dehydroascorbic acid reductase found in plants, is involved in the reduction of DHA by human erythrocytes. *Parrot* and *Cotereau* (1957) state that vitamin P causes an improved utilization of vitamin C by the organism, probably by catalyzing the reduction of DHA to AA by glutathione.

Kellie and *Zilva* (1935), *van Eekelen* (1936) and *Fujita* and *Ebihara* (1937) have reported that AA is oxidized by oxyhemoglobin, and that this results in a decrease of the amount of AA when whole blood is analyzed. *Fischer* (1937) and *Gabbe* (1937), on the other hand, report that AA in the plasma is absorbed by oxyhemoglobin. *Fujita, Ebihara* and *Numata* (1939) could not confirm these results, and state that AA is not oxidized directly by oxyhemoglobin, but when the precipitating agents, such as trichloracetic acid or metaphosphoric acid are added to the sample, the oxyhemoglobin breaks down, and the molecular oxygen liberated causes the oxidation of AA. A more recent report by *Vladimirov* and *Kolotilova* (1947) suggests that after the acid treatment of the blood sample, the denaturated product of oxyhemoglobin either acts directly, or catalyzes the oxidation of AA by the oxygen of the air.

The oxidation of AA in whole blood can be prevented by saturation of the blood with carbon dioxide, carbon monoxide or hydrogen cyanide (*Kellie* and *Zilva* 1935, *van Eekelen* 1936, *Butler* and *Cushman* 1939, *Fujita, Ebihara* and *Numata* 1939 and *Vladimirov* and *Kolotilova* 1947).

Christine et al. (1956) observed a reduction of DHA when added to a hemolysed suspension of erythrocytes saturated with carbon monoxide. The author, on the other hand, observed an

oxidation of AA to DHA when added to hemolysed blood not saturated with carbon monoxide.

In the present investigation, the main problem was the passage of AA and DHA from the mother to the fetus, and the relative changes of concentration were of more importance than the exact differentiation of the different forms of the vitamin within the cells. Only TAA was measured in whole blood according to the method previously described. The TAA in the cells was calculated from the formula

$$A_c = (A_{wb} \cdot 100 - A_p \cdot V_p) / V_c$$

where A_c, A_{wb} and A_p represent the concentrations of TAA in the cells, the whole blood and plasma respectively, and V_p and V_c represent the plasma and the cell volumes respectively. A_c thus refers to the total red and white cell ascorbic acid.

IV. Transfer of vitamin C from plasma to erythrocytes

1. Introduction

It has long been known that the mammalian erythrocyte behaves as an osmometer, and it has therefore been extensively used in the studies of membrane permeability to both electrolytes and nonelectrolytes.

The earliest reports in the literature relating to the transfer of vitamin C from plasma to erythrocytes are those of *Borsook* et al. (1937) and *Heinemann* (1938). Borsook et al. observed that AA added to human whole blood *in vitro* remained in the plasma, and concluded that the red cells were in the main, if not absolutely, impermeable to added AA. Heinemann, on the other hand, concluded from *in vitro* and *in vivo* experiments that added AA passed from the plasma into human red cells. The *in vivo* experiments of *Butler* and *Cushman* (1940) showed a slow penetration of AA into human erythrocytes. *Heinemann* and *Hald* (1940), in studies on the factors which influence the

passage of AA from plasma to cells in human blood, found that the vitamin entered the cells at 37°C, but not at 7°C.

In all the above mentioned studies only the reduced form of ascorbic acid, AA, was determined, and no attention was paid to a possible difference in behaviour of the oxidized form of the vitamin, DHA.

Panteleeva (1950) was the first to differentiate between the penetration of AA and DHA into the erythrocytes *in vitro*. She observed a slow permeation of AA into the erythrocytes of man, horse, cat and rabbit. DHA, on the other hand, penetrated human erythrocytes very rapidly, attaining a much higher concentration than in the plasma. With the horse, pig, dog, cat and rabbit, however, the plasma remained richer in DHA. After permeation into human or animal erythrocytes, DHA was rapidly reduced to AA. The author concluded that the exchange of vitamin C between the plasma and the erythrocytes occurs mainly in the form of DHA.

Lloyd (1951) and *Lloyd* and *Parry* (1954) report similar results from *in vitro* experiments on human blood. *Golden* and *Sargent* (1952) state that AA does not enter human erythrocytes *in vitro*, although a slow transfer is observed *in vivo*. In a recent study, *Christine* et al. (1956) showed that DHA easily penetrates into human erythrocytes, where it is reduced by a still unknown mechanism, and that AA on the other hand, penetrates the erythrocytes very slowly.

The normal distribution of TAA, AA and DHA between plasma and cells has been studied by several workers (*Butler* and *Cushman* 1940, *Crandon, Lund* and *Dill* 1940, *Heinemann* 1938, *Lowry* et al. 1946, *Roe, Kuether* and *Zimler* 1947 and *Sargent* 1947). The lack of agreement in the obtained data indicates that the results depend to a great extent on the analytical methods used for the determination of the vitamin. This has been shown recently by *Iggo, Owen* and *Stewart* (1956).

The object of the present study was to investigate and compare the *in vitro* passage of AA and DHA from plasma to erythrocytes in adult and fetal blood of man and guinea pig, in order to clarify the results of the *in vivo* experiments on placental transfer.

2. Method

A study was made of the behaviour of AA and DHA after their addition to whole blood *in vitro*. AA or DHA was added, in quantities ranging between 2 and 4 mg per 100 ml blood, as successive additions at 20 minute intervals, and the samples were incubated at room temperature. Samples for the determination of TAA in plasma and whole blood were collected just prior to every addition. The penetration of DHA into erythrocytes was compared in adult and fetal blood samples of man and guinea pig. The mechanism of transfer of DHA through the erythrocyte membrane was elucidated by experiments in which the blood was incubated at 0°C, 23°C and 37°C. The effect of temperature on the permeability was calculated as the temperature coefficient Q_{10} from the formula $\text{Conc.}_{(T+10)}/\text{Conc.}_T$. The role of free —SH groups in the permeability of the erythrocyte membrane to DHA was studied by the use of $HgCl_2$ as an —SH inhibitor.

3. Results

After successive additions of AA to adult and fetal human whole blood at room temperature, nearly all of the added vitamin, upon analysis, was found in the plasma, the erythrocytes showing only a very slight increase in TAA (figure 2).

The *in vitro* permeability of adult and fetal human erythrocytes to DHA was studied in venous blood samples from four healthy adults, and from the cord blood of four normal deliveries. The results of one typical experiment in each group are shown in figure 3. After successive additions of DHA in the quantities indicated in the figure, a marked increase of TAA was found in the erythrocytes, indicating a rapid penetration of the added DHA through the erythrocyte membrane. The rate of permeation of DHA into adult and fetal human erythrocytes occurred at equal rates (figure 3).

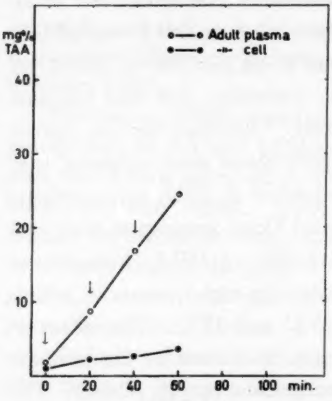


Figure 2. Change in the concentration of total ascorbic acid in plasma and cells after additions of 4 mg of L-ascorbic acid per 100 ml adult human whole blood at the points indicated in the figure by arrows.

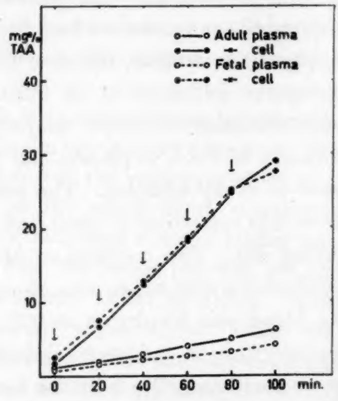


Figure 3. Change in the concentration of total ascorbic acid in plasma and cells after additions of 3.75 mg of dehydroascorbic acid per 100 ml adult human whole blood, and 3.43 mg of dehydroascorbic acid per 100 ml fetal human whole blood at the points indicated in the figure by arrows.

In order to compare the behaviour of the erythrocytes of adult and fetal guinea pigs with that of human blood, four pregnant animals were sacrificed at the end of gestation, and DHA additions as above were made to the collected samples of maternal and fetal blood. The results of a typical experiment on each type of blood can be seen in figure 4. It was found that DHA penetrated fetal guinea pig erythrocytes at a rate corresponding to that of human blood, whereas the passage of DHA into maternal guinea pig erythrocytes occurred more slowly. In one case a slow accumulation of TAA was found in the maternal cells, in two cases the plasma remained richer, and in the last

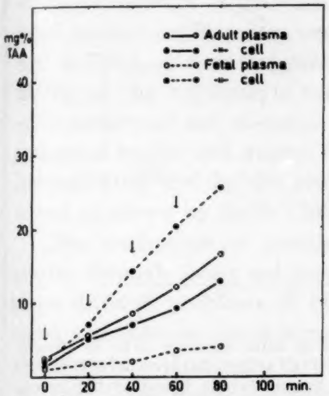


Figure 4. Change in the concentration of total ascorbic acid in plasma and cells after additions of 3.35 mg of dehydroascorbic acid per 100 ml maternal and fetal whole blood of guinea pigs at the points indicated in the figure by arrows.

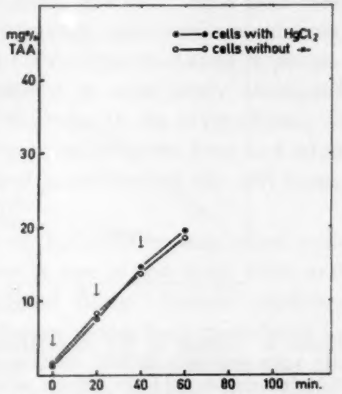


Figure 5. Change in the concentration of total ascorbic acid in adult human washed erythrocytes after additions of 3.0 mg of dehydroascorbic acid per 100 ml with and without the presence of 2×10^{-5} M of $HgCl_2$.

experiment an equilibrium was established between the extracellular and the intracellular concentration of the vitamin.

The results seen in figure 5 show that the presence of 2×10^{-5} M of $HgCl_2$ in a suspension of washed human erythrocytes in physiological saline did not alter the rate of transfer of DHA into the cells.

The temperature coefficient Q_{10} of the rate at which the erythrocytes take up DHA is indicated by a comparison of the slopes of the curves in figure 6. The Q_{10} calculated from experiments in which human blood was incubated at 0°C, 23°C and 37°C, after additions of 2 mg of DHA per 100 ml blood was about 1.2.

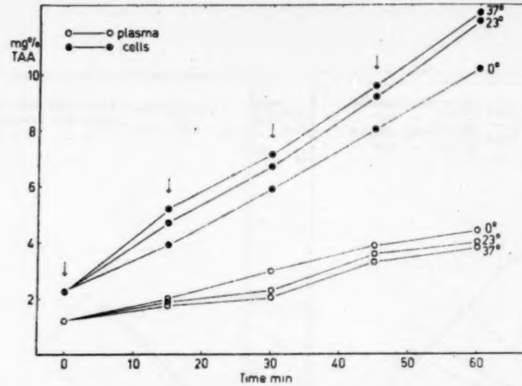


Figure 6. Change in the concentration of total ascorbic acid in plasma and cells incubated at 0°C, 23°C and 37°C after additions of 2.0 mg of dehydroascorbic acid per 100 ml adult human whole blood at the points indicated in the figure by arrows.

4. Discussion

The above experiments *in vitro* confirm earlier reports which state that DHA easily penetrates the human erythrocyte membrane, whereas AA penetrates very slowly if at all (*Panteleeva* 1950, *Lloyd* 1951, *Lloyd* and *Parry* 1954 and *Christine* et al. 1956). The slight increase in the concentration of TAA which was observed in the cells after the addition of AA (figure 2) was most probably due to a slow oxidation of the added AA to DHA, which in its turn entered the erythrocytes. Such an oxidation has been found to occur in plasma *in vitro* (*Borsook* et al. 1937, *Stotz* et al. 1937—1938 and *Lowry, Lopez* and *Bessey* 1945). The results of *Heinemann* and *Hald* (1940) that ascorbic acid enters the blood cells at 37°C but not at 7°C are in agreement with this assumption. Any enrichment of DHA in the plasma would be hard to detect because of its rapid penetration into the cells.

The difference in the rate of taking up DHA found between the adult and the fetal erythrocytes of the guinea pig can be compared to the results of *Widdas* (1954, 1955) according to

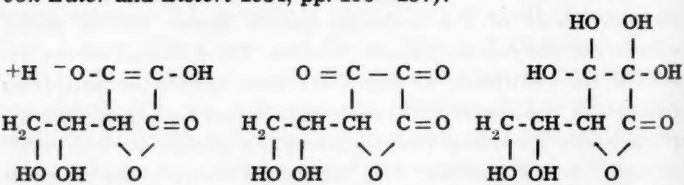
which the erythrocytes of the fetuses of such animals as the pig, sheep, deer, rabbit and guinea pig are permeable to glucose, whereas the cells of their adult forms are not. Both adult and fetal human erythrocytes are, however, permeable to glucose, and to DHA, as seen in figure 3. This type of change in permeability of the erythrocyte membrane is most likely associated with structural and chemical differences in the erythrocytes, as indicated by the well known differences between fetal and adult hemoglobins, and by the physical properties of the cell membrane as shown by *Sjölin* (1954).

The mechanism of transfer of biologically important molecules through living membranes is one of the most basic and most difficult problems in biological study. Several explanations and theories for this mechanism have been postulated in the literature of recent years. *Danielli* (1954a, 1954b) suggests at least three kinds of process by which transfer may occur. Firstly, it may occur by *simple diffusion*, which is brought about by molecular agitation. In this process, the only selectivity is in terms of molecular size, and structural and steric factors are not involved. Simple diffusion may occur alone, or in conjugation with facilitated diffusion or active transport. In *facilitated diffusion*, the structural and steric factors are important, and the movement of the molecule occurs across certain areas, »pores», of the cell membrane, whereas the diffusion across the rest of the membrane is slow. At these pores, the activating energy for the membrane penetration is lowered in some way, possibly by providing hydrogen-bonding groups or free —SH groups. *Active transport* is a mechanism whereby the molecule is moved against a concentration gradient at the expense of energy derived from the metabolism of the cell.

The increase in the concentration of TAA in the erythrocytes after additions of DHA to the blood involves the passage of the DHA molecule through the cell membrane, and its reduction to AA. Within the range of the DHA concentration used, the passage through the cell membrane, as compared with the reduction, seems to occur much more slowly, and it is therefore most probable that the rate of transfer is limited only by the speed of permeation of the DHA molecule through the erythrocyte cell

membrane. Hence, DHA never passes against a concentration gradient and since the temperature coefficient of the transfer is of the order of 1.2 it is most likely that the rate of transfer is controlled by a mechanism other than the energy requiring enzymatic processes of cell metabolism. As a comparison, it can be mentioned that the temperature coefficient of the rate at which human erythrocytes take up glucose is about 2.5 (*Bjering* 1933 and *Le Fevre* 1948), and 5.5 with smaller glucose concentrations (*Bang* and *Örskov* 1937).

The explanation for the striking difference in permeability of the erythrocyte to AA and to DHA must be found in the difference in molecular structure and chemical behaviour of the two forms of the vitamin. In aqueous solution AA behaves as a monobasic acid, forming salts containing one monovalent metal atom or equivalent. The hydrogen atom of the enol group on carbon atom 3 dissociates yielding a solution with a pH of about 3. In alkaline solution, however, the enol group on carbon atom 2 also dissociates. AA can thus be considered as an electrolyte, whereas its oxidized form DHA is a neutral substance, a γ-lactone and a nonelectrolyte, which in aqueous solution is most likely hydrated (*Roe* 1954, p. 117, *Smith* 1954, p. 185, and *von Euler* and *Eistert* 1954, pp. 196—237).



L-ascorbic acid — Dehydroascorbic acid — Hydrated form of dehydroascorbic acid

Le Fevre (1948) showed that the permeability of human erythrocytes to glucose is extremely sensitive to —SH inhibitors such as Hg^{++} and p-chloromercuribenzoate in concentrations as low as $1—5 \times 10^{-6}$M. The effect of these —SH inhibitors is prevented or reversed in the presence of glutathione or cysteine. He suggested a participation of —SH groups at the cell surface as an essential step in the passage of glucose and other like substances across the human red cell membrane.

Demis and *Rothstein* (1955) found a rapid inhibition by $HgCl_2$ of glucose uptake by intact rat diaphragm.

The results of the present investigation show that the transfer of DHA is unaltered by the presence of Hg^{++} in a concentration of 2×10^{-5}M, an observation which speaks against any participation of —SH groups at the cell surface in the transfer of DHA through the human erythrocyte membrane.

V. Transfer of vitamin C across the guinea pig placenta

1. Introduction

Placental transfer of nutrients, especially that of sugars, has been extensively studied during recent years. For a review of literature on this subject, see *Huggett* (1956) and *Snoeck* (1958).

The author has been unable to find any literature referring to direct experimental studies on the placental transfer of vitamin C in the guinea pig or in any other animal.

2. Methods

The experimental technique used in the following experiments has been previously described in chapter III (page 9).

3. Results

Transfer of L-ascorbic acid from mother to fetus. — 14 experiments were performed, the experimental data and results of which are to be seen in table III (appendix). In the preliminary experiments T_1 to T_7 only the changes in TAA were estimated in the maternal and fetal plasma. A slight increase was observed in the fetal plasma during the infusion of 2 to 6 mg AA into the maternal circulation. In experiments T_{12} and A_1, the changes of TAA were estimated in plasma and cells, and in the remaining experiments, A_4, A_5, A_7, A_9 and A_{12}, the changes of AA

and DHA were estimated in plasma, in addition to TAA in plasma and cells. The changes of TAA in the amniotic fluid were studied during the infusion in experiments A_4, A_5, A_7 and A_9. In these experiments, one or two fetuses were left untouched in the uterus, and samples of amniotic fluid were collected by puncture of the fetal membranes. The amniotic fluid showed very slight or no increase of TAA during the experiments in which AA was infused.

In experiments A_4 and A_5, a marked degree of hemolysis was found in the maternal blood, due to an accidental infusion of an aqueous solution of AA. As previously mentioned, hemolysed blood which is not saturated with carbon monoxide will oxidize AA to DHA, and thus a marked increase in the concentration of DHA in the maternal blood was found in experiments A_4 and A_5. The subsequent greater increase of TAA in the fetal blood in these experiments, as compared with the experiments in which no hemolysis occurred, together with the results of the *in vitro* experiments on erythrocyte permeability, strongly suggested that the placenta behaves as the erythrocyte, being permeable to DHA and impermeable or almost impermeable to AA.

An example of a typical experiment (A_{12}), in which 20 mg AA was infused into the maternal circulation, is presented in figure 7. The increase of TAA on the fetal side of the placenta is only very slight. It is evident that both the maternal and the fetal plasma contain more TAA than do the cells. At the end of the experiment, the maternal plasma DHA equals the TAA of the fetal plasma. This also is in agreement with the assumption that the increase of TAA in the fetal blood during the infusion of AA is due to a transfer of DHA formed in the maternal organism from the infused AA.

Transfer of dehydroascorbic acid from mother to fetus. — 9 experiments were performed, as shown in table III. In experiments T_8 to T_{11}, DHA was infused into the mother in amounts ranging from 4 mg to 10 mg. These amounts were insufficient to produce any noticeable increase on the fetal side, since most of the DHA infused must have passed into the maternal tissues

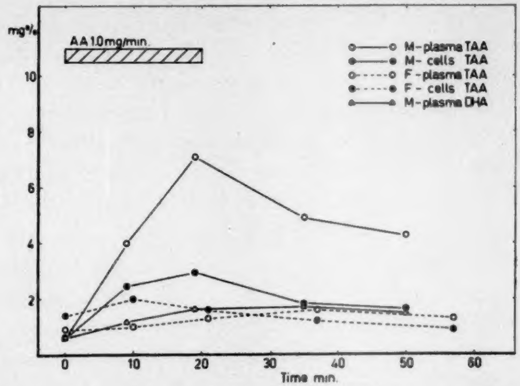


Figure 7. Infusion of 1.0 mg of L-ascorbic acid per min during 20 minutes into the guinea pig mother (M). The maternal plasma shows a marked increase in the concentration of total ascorbic acid during the infusion but the increase in the fetal (F) plasma and cells is only slight. At the end of the experiment the maternal plasma concentration of dehydroascorbic acid equals the total ascorbic acid concentration in the fetal plasma.

before reaching the placenta, as is seen from the low maternal plasma values of TAA. When the amount of DHA in the infused solution was increased to a total ranging between 19 and 24 mg in experiments A_2, A_3, A_6, A_{10} and A_{14}, a marked transfer across the placenta could be observed (table III). Figure 8 shows the results of a typical experiment (A_{10}), in which 21 mg DHA was infused into the mother. The fetal plasma shows only a slight increase of TAA, whereas the erythrocytes rapidly take up the vitamin, as has already been shown in the *in vitro* experiments on blood. Again, at the end of the experiment, the DHA concentration of the maternal plasma equals the TAA concentration of the fetal plasma, whereas the fetal cells show a marked accumulation of TAA.

Two experiments[1] were performed in which one fetus was cooled to about +27°C whereas the other was kept at +37°C. in order to compare the transfer of DHA across the placenta at different temperatures. Figure 9 shows the results of one of

[1] The results of one of these experiments are seen in figure 9. During the other experiment the photograph shown in figure 10 was taken and due to the incautious handling of the fetuses the placental blood flow was altered, and the results are thus unreliable.

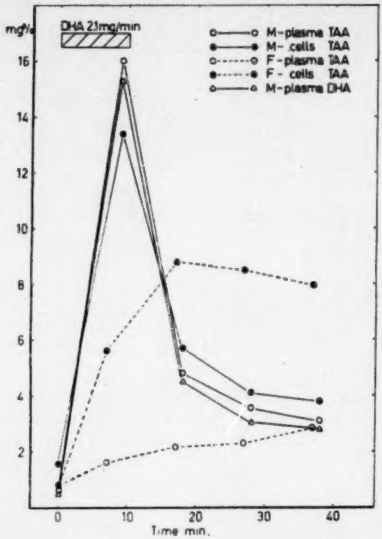


Figure 8. Infusion of 2.1 mg of dehydroascorbic acid per min during 10 minutes into the guinea pig mother (M). Both maternal plasma and cells show a rapid increase in the concentration of total ascorbic acid during the infusion. The fetal (F) plasma shows a slight increase of total ascorbic acid, but a marked accumulation is found in the cells. At the end of the experiment the maternal plasma concentration of dehydroascorbic acid equals the total ascorbic acid concentration in the fetal plasma.

these experiments. The rate of change in the TAA concentration is slightly greater in the blood of the cold fetus as compared with the rate in the warm fetus. At the end of the experiment, the cold fetus contained 14.7 mg TAA per 100 g fetal tissue, and the warm fetus contained 13.4 mg per 100 g.

A rather small but nevertheless clear increase of TAA was found in the amniotic fluid during the infusion of DHA in experiments T_8, A_2, A_3 and A_6 (table III).

4. Discussion

From the above experiments, it is evident that DHA passes through the placental barrier from the maternal plasma to the

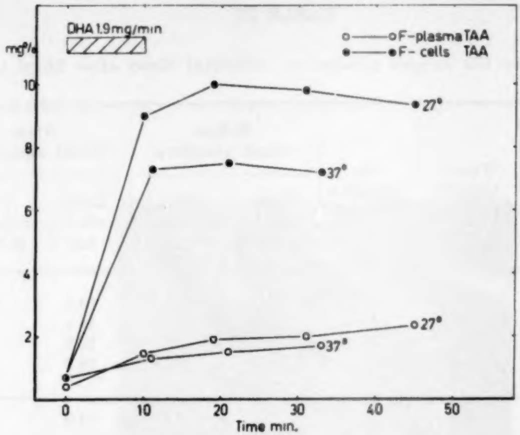


Figure 9. **Change in the concentration of total ascorbic acid in plasma and cells of two guinea pig fetuses kept at 27°C and 37°C during the infusion of 1.9 mg of dehydroascorbic acid per min into the maternal circulation.**

fetal plasma, and further into the fetal erythrocytes, as already shown in the *in vitro* experiments on blood.

On the other hand, AA infused into the maternal circulation does not penetrate the placenta, or penetrates it very slowly. Whether the slight increase of TAA found in the fetal blood is due to a transfer of AA or of DHA, e.g. infused AA oxidized in the maternal organism, is difficult to prove by ordinary chemical analysis. The experiments in which the maternal blood was hemolysed, however, strongly support the view that the increase of TAA in the fetal blood during the infusion of AA is due to a transfer of DHA through the placenta.

It has been shown that the permeability of placenta to a series of sugars is essentially analogous to the permeability of human erythrocytes to different pentoses and hexoses (*Karvonen* and *Räihä* 1957). The present investigation indicates that a similar analogy exists for the transfer of vitamin C.

The interpretation of the results of the experiment in which

TABLE IV

Change in the oxygen content of umbillical blood after blood loss.

No. of Exp.	Fetal weight in g	O_2 capacity in vol %	Before blood sampling		After blood sampling	
			Umb. vein O_2 in vol %	Umb. artery O_2 in vol %	Umb. vein O_2 in vol %	Umb. artery O_2 in vol %
1	75	19.7	6.0	2.5	14.7	3.6
2	78	19.8	9.9	2.1	15.1	2.8
3	80	20.1	11.7	2.5	15.8	4.2
4	90	20.0	10.3	2.2	14.5	2.9
	Mean	19.9	9.5	2.3	15.0	3.4

the effect of temperature on the rate of placental transfer of DHA was studied is not so simple as that of the *in vitro* experiments with erythrocytes, since any change in the placental circulation of the cold fetus must be considered. Figure 10 shows the difference in color between the warm and the cold placenta, the cold being red and the warm bluish. Determinations of the oxygen saturation showed a 50 % saturation in the umbilical vein of the warm fetus, and a 75 % saturation in the cold one. A similar increase of the oxygen saturation of the umbilical vein was obtained after several blood samplings from the fetus, decreasing its total blood volume by ca. 3 ml (table IV).

Although no direct conclusions can be drawn from the results of the above experiments concerning a change in the rate of transfer of DHA across the placental barrier in the cold fetus, it would seem highly improbable that an increase in the rate of permeability of vitamin C has occurred. The marked increase in the oxygen saturation (about 50 %), as compared with the relatively small increase in the TAA content of the blood in the umbilical vein of the cold fetus could hence be due to a com-

Figure 10. **The red placenta of a guinea pig fetus kept in physiologic saline at 27°C, and the bluish placenta of one kept at 37°C.**

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bined effect of a slower blood flow in the cold placenta (see *Barcroft* 1946, p. 190) and a decreased oxygen consumption of the cold fetus.

Another explanation would be the existence in the guinea pig of an »extra-villous shunt» mechanism, as described by *Böe* (1954) in the human. It can be postulated, that the amount of blood which would flow through this shunt would decrease in the cold placenta, causing a marked increase in the oxygen content, but only a slight increase in the TAA content of the blood in the umbilical vein. *Burwell* (1955, p. 203) discussed the influence of such a mechanism at the Second Macy Conference on Gestation: »It is obvious that one of the figures that can affect this calculated pressure gradient is the volume of fetal blood that goes through the placenta without coming into effective relation with maternal blood. If there is a considerable volume of such fetal blood, this will influence the oxygen tension of the blood going from the placenta to the fetus and, therefore, affect this calculated pressure gradient.» Such a shunt mechanism would also explain the findings of *Barron* and *Meschia* (1954), that the carbon dioxide tension in the sheep is always higher on the fetal side of the placenta, despite the high diffusion constant of carbon dioxide. It would be possible to verify the existence of such a shunt mechanism by demonstration of a decrease in the carbon dioxide tension on the fetal side of the placenta when cooling a fetus.

VI. Transfer of vitamin C across the human placenta

1. Introduction

The plasma vitamin C content of the newborn infant, and of cord blood, has been shown to be considerably higher than that of the maternal blood (*Wahren* and *Rundqvist* 1937, *Neuweiler* 1938, *Braestrup* 1938, *Teel, Burke* and *Draper* 1938, *Manahan* and *Eastman* 1938, *Elmby* and *Becker-Christensen* 1938, *Braestrup* 1939, *Snelling* and *Jackson* 1939, *Möller-Christensen* and *Thorup* 1940, *Mindlin* 1940, *McDevitt* et al. 1942, *Lund* and *Kimble* 1943, *Slobody, Benson* and *Mestern* 1946 and *Hamil* et al. 1947). The first urine passed after birth contains a high concentration of vitamin C, followed by a decrease in the concentration in the fetal tissues (*Ingalls* 1938, *Hamil* et al. 1947).

The high vitamin C values of fetal blood, even in cases where the maternal vitamin C nutrition is poor, have puzzled investigators, and several interesting suggestions have been offered. *Giroud* (1936) attributed it to the ability of the fetus to synthesize vitamin C. *Manahan* and *Eastman* (1938) presented the idea that »selective filtration» of ascorbic acid occurs in the placenta. *Smith* (1939), on the other hand, held that the depressing effect of exercise, of sweating in labour, and of anesthesia, led to a loss of vitamin C by the maternal organism. *McDevitt* et al. (1942) confirmed the observations of Manahan and Eastman, and agreed that the placenta acts as a selective filter at all levels of vitamin C deficiency or saturation of the mother. *Lund* and *Kimble* (1943) found that administration of vitamin C to the mother parenterally caused an elevation to equal levels of the values for both maternal and fetal plasma.

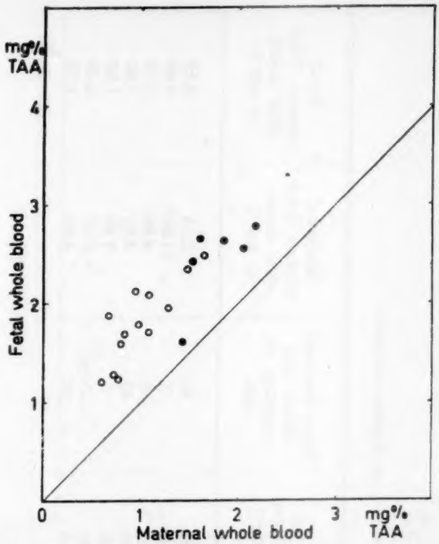

Figure 11. The relation between the concentration of total ascorbic acid in maternal and fetal human whole blood. The black points indicate the cases in which L-ascorbic acid has been administered *per os* to the mother during some days before delivery.

Rapid reduction of the maternal level to normal followed, while the fetal values remained high. The authors concluded that the placenta acts as a barrier to the re-entry of ascorbic acid into the maternal circulation, and they expressed the belief that this mechanism of »selective retention» would account for the difference between maternal and fetal vitamin C values.

2. Method

The human material, and the experimental technique used, have been previously described in chapter III (page 9).

3. Results

Table V shows the experimental data, and the results of the experiments on the human subjects during legal abortion. A

TABLE V

Experimental data of the experiments on human subjects.

Exp.	Fetal weight in g	Maternal plasma TAA before inject. in mg %	Fetal plasma TAA before inject. in mg %	Dose of AA injected in mg	Time between inject. and sampling in min	Maternal plasma TAA after inject. in mg %	Fetal plasma TAA after inject. in mg %
B. S.	550	0.80	—	500	30	2.10	4.40
A. F.	500	0.50	1.00	1000	3	11.00	2.00
D. V.	150	0.55	—	900	19	4.00	1.70
F. Y.	500	1.00	—	1000	40	3.65	3.80
B. R.	350	0.70	1.05	1000	6	7.60	1.90
K. K.	136	0.60	1.20	1500	15	8.00	3.20
S. Ö.	240	0.30	—	800	16	4.50	1.70

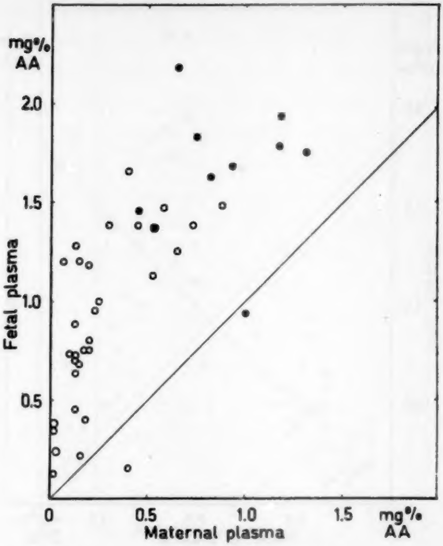

Figure 12. The relation between the concentration of L-ascorbic acid in maternal and fetal human plasma. The black points indicate the cases in which L-ascorbic acid has been administered *per os* to the mother during some days before delivery.

clear increase of TAA is seen in the fetal plasma after the injection of AA into the maternal circulation, and particularly in experiments B.S. and F.Y., in which the time between the injection and the collection of blood from the umbilical vein exceeded 30 minutes.

The sample of DHA used in this investigation was considered to be unsuitable for injection into human subjects.

Figure 11 shows the distribution of TAA between maternal whole blood and cord blood in 19 cases. The black points mark the cases in which AA was administered to the mother before delivery. The TAA content of the cord blood has a mean value of 2.01 mg per 100 ml, and is in all cases considerably higher than the concentration in the maternal whole blood, which has a mean of 1.24 mg per 100 ml.

Figures 12 and 13 respectively show the distribution of AA

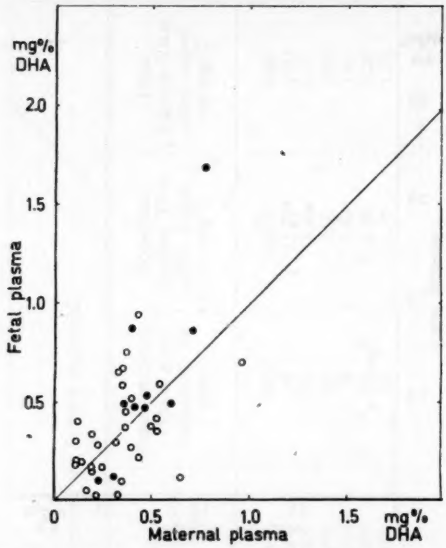

Figure 13. The relation between the concentration of dehydroascorbic acid in maternal and fetal human plasma. The black points indicate the cases in which L-ascorbic acid has been administered *per os* to the mother during some days before delivery.

and DHA between maternal and fetal plasma obtained during 41 normal deliveries. DHA is distributed more equally on both sides of the placenta, having a mean concentration of 0.38 mg per 100 ml in the maternal plasma, and 0.40 mg per 100 ml in the fetal plasma. AA, on the other hand, is clearly retained in the fetal plasma, having a mean concentration of 1.06 mg per 100 ml, whereas the maternal plasma shows a mean concentration of 0.40 mg per 100 ml.

When the content of TAA of the amniotic fluid was studied, and related to that of the maternal or fetal plasma, it became evident that when the maternal plasma TAA exceeded the mean normal value [1] of 0.64 mg per 100 ml, an accumulation of TAA occurred in the amniotic fluid (figure 14). When, on the other

[1] The mean concentration of TAA in the plasma of the mothers who did not receive vitamin C in the hospital prior to delivery.

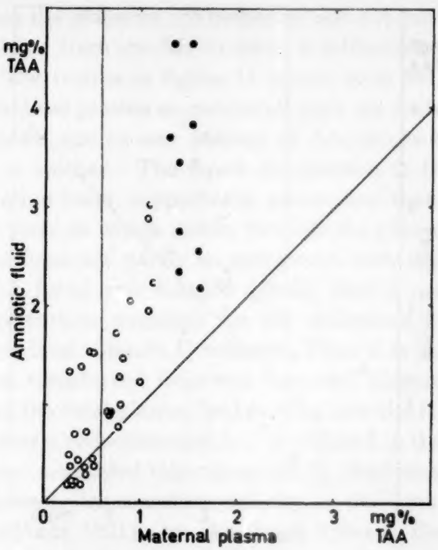

Figure 14. The relation between the concentration of total ascorbic acid in maternal human plasma and amniotic fluid. The vertical line indicates the mean normal maternal plasma value, and the black points the cases in which L-ascorbic acid has been administered *per os* to the mother during some days before delivery.

hand, the fetal plasma TAA showed values below the mean normal value [1] of 1.24 mg per 100 ml, the amniotic fluid became depleted of its TAA and showed values below 1.24 mg per 100 ml (figure 15).

4. Discussion

From the results obtained by *Clayton, McSwiney* and *Prunty* (1954) relating to the metabolism of DHA in the human, it can be seen that after an intravenous injection of 200 mg of AA the difference between plasma AA + DHA and plasma AA increases slightly during the two hours immediately following the injec-

[1] The mean concentration of TAA in the plasma of the fetuses whose mothers did not receive vitamin C in the hospital prior to delivery.

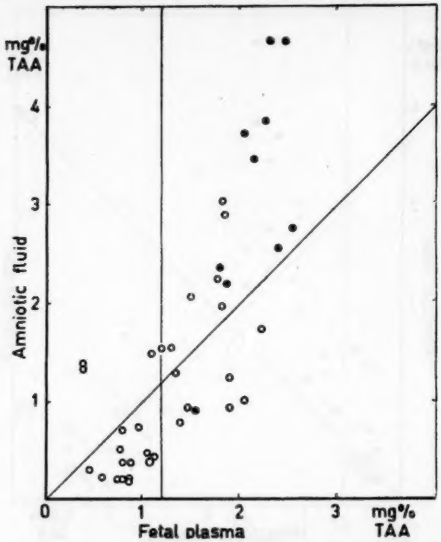

Figure 15. The relation between the concentration of total ascorbic acid in fetal human plasma and amniotic fluid. The vertical line indicates the mean normal fetal plasma value, and the black points the cases in which L-ascorbic acid has been administered *per os* to the mother during some days before delivery.

tion. In the present investigation, however, no increase in the plasma DHA was found after an intravenous injections of 500 mg of AA into two adult male subjects, neither could any increase of the TAA in the erythrocytes be observed. In studies on the metabolic products of L-ascorbic acid 1-C^{14} in preparations of guinea pig liver *Chan, Becker* and *King* (1958) have shown that DHA is a major intermediate.

The increase of TAA found in the plasma of the human fetuses after intravenous injection of AA to the mothers is of the same order of magnitude as that found in the experiments conducted on the guinea pigs during infusion of AA. The possibility that the plasma DHA of the pregnant mothers shows an elevation during the intravenous administration of AA makes it possible to assume that DHA oxidized from the injected AA has

passed across the placenta. Whether or not AA has crossed the human placenta from mother to fetus is difficult to verify with certainty. The results in figure 11 which show the high levels of AA in the fetal plasma as compared with the maternal levels, however, speak against any passage of AA across the placenta from fetus to mother. The equal distribution of DHA (figure 12), on the other hand, supports the assumption that DHA is the form of the vitamin which passes through the placental barrier.

These findings are partly in agreement with the hypothesis presented by *Lund* and *Kimble* (1943), that a mechanism of »selective retention» accounts for the difference between the maternal and fetal vitamin C values. Thus it is fairly evident that DHA is transferred from the maternal plasma across the placenta into the fetal plasma, and further into the fetal erythrocytes, and after a reduction to AA it is retained in the fetus.

It has been suggested that the amniotic fluid may be formed partly or entirely by secretory activity of the amniotic membrane (*Needham* 1931), by the fetal kidney (*Bernstine* and *Meyer* 1953 and *McCance* and *Widdowson* 1954), by the fetal lungs (*Dawes* 1954 and *Jost* 1954) and in the buccal and nasal cavities (*Reynolds* 1953). Whatever the source of the amniotic fluid may be, it is evident from the results shown in figure 14 that when vitamin C is administered to the mother some time before delivery, an accumulation of the vitamin is observed in the amniotic fluid. The animal experiments on the other hand showed that DHA infused into the mother produced an increase of TAA in the amniotic cavity, whereas the infusion of AA did not alter the concentration of the vitamin in the amniotic fluid during the experiment.

Recently, *Garby* (1957) presented a comprehensive study on the exchange of various molecules and ions across the isolated human amniotic membrane. He concluded that although the net liquid flow across the amniotic membrane is small, of the order of 0.3—3.0 ml/hr in a direction out of the amniotic cavity, the net transfer of some substances may have a physiological significance. NaCl, KCl and glucose flow into the amniotic cavity, whereas urea and creatinine flow out of the cavity. *Paul* et al. (1956) showed that in the rabbit about one half of the

exchange of water occurs across the membranes by way of the maternal circulation, and one half occurs by way of the placenta and fetus. Whether the vitamin C found in the amniotic fluid is excreted by the fetal kidney, or is transferred through the membranes, cannot be determined on the basis of these experiments. It is, however, known that in adults vitamin C is mainly excreted in urine as AA (*Hellman* and *Burns* 1958). It has also been shown that amniotic fluid is swallowed by the fetus and absorbed by the fetal digestive tract and respiratory tract (*Piaszek* 1947, *Kraus* 1951).

It can thus be postulated that when the maternal vitamin C nutrition is good, a considerable amount of the vitamin crosses the placenta as DHA and is excreted by the fetus into the amniotic cavity, or passes directly through the membranes into the amniotic fluid, where it accumulates, and from where it can be reabsorbed through the fetal digestive tract during times of poor nutrition when the TAA in the fetal blood tends to decrease.

VII. General discussion

The role of vitamin C in the different biochemical processes in which it participates is outside the scope of this discussion, which deals primarily with the possible significance of the high fetal vitamin C values, and the mechanism of placental transfer by which this concentration gradient is obtained and maintained. For a recent review on the functions of vitamin C in biochemical systems, see *Mapson* (1954).

It has been shown that the oxidized form of the vitamin, — dehydroascorbic acid — rapidly passes across the placenta from mother to fetus in the guinea pig, whereas the reduced form of the vitamin — L-ascorbic acid — is transferred very slowly if at all. The results of the studies on human material indicate that the transfer of vitamin C across the placenta most probably occurs as dehydroascorbic acid, and that the high content of vitamin C in fetal blood is mainly due to an increase in

the concentration of L-ascorbic acid on the fetal side of the placenta. This mechanism of transfer would make it possible for the fetus to maintain a high concentration of vitamin C. *In vitro* experiments on adult and fetal blood of human subjects and of guinea pigs showed that dehydroascorbic acid rapidly passes from plasma to erythrocytes, whereas L-ascorbic acid remains in the plasma when added to the blood. Whether the transfer of vitamin C across other biological membranes occurs in the form of dehydroascorbic acid is a question which needs further investigation. The studies of *Kinsey* (1950) and of *Barany* and *Langham* (1955), however, indicate that the passage of vitamin C across the blood-aqueous humor barrier does not occur as dehydroascorbic acid. Injected directly into the aqueous humor, dehydroascorbic acid became reduced only slowly, and when the concentration of dehydroascorbic acid in the plasma was increased, the L-ascorbic acid concentration in the aqueous humor did not increase above that corresponding to the prevailing concentration of L-ascorbic acid in the plasma.

It is well known that the latter part of pregnancy is accompanied by a physiological state of hypoxia of the fetus (*Barcroft* 1946). *Galperina* (1951) has shown that an introduction of ascorbic acid into the rabbit fetus, or to the mother, leads to a prolongation of life of the former after tying the umbilical cord. *Krauso* et al. (1950) have found that repeated exposure of human subjects to 18,000 feet without supplemental oxygen resulted in a continuous drop in their ascorbic acid excretion. These findings suggest that more vitamin C is required for the state of low oxygen tension of the blood, and that vitamin C might have a function when the metabolic organization of the organism is more anaerobic in character.

In close agreement with the above suggestion are the results of *Stern* and *Timonen* (1954), in their study on the metabolism of the cell nucleus of thymus and liver tissue. These authors found that the cytochrome oxidase system and the system of flavoproteins are both lacking in the nucleus, and concluded that nuclear metabolism must be anaerobic. It was suggested as highly probable that ascorbic acid acts as a mechanism of hydrogen transport in the energetic metabolism of the

nucleus, and would be especially required during the process of chromosome duplication. It was also possible for the above authors to demonstrate a close association between the ascorbic acid concentration and the mitotic process in lily anthers.

The outstanding characteristic of prenatal life is fast growth, including differentiation and initiation of new structures and functions. Thus, if vitamin C is involved in nuclear metabolism and in the process of chromosome duplication, it is evident why the developing fetus needs such a great tissue saturation of ascorbic acid. The observations mentioned in the introduction on the frequency of prematurity, stillbirth, retarded fetal growth and development caused by vitamin C deficiency, are all in agreement with the above presented idea.

VIII. Summary

The purpose of the experimental investigation presented here, was that of studying the process of transfer of vitamin C across the placental barrier from mother to fetus in guinea pigs and in human subjects.

1. Due to the numerous chemical methods which have been suggested in the literature, for the determination and the differentiation of total ascorbic acid, L-ascorbic acid and dehydroascorbic acid in plasma and whole blood, and to the conflicting reports concerning the existence of dehydroascorbic acid in biological material, preliminary studies were undertaken to find suitable analytical methods for the present investigation. These studies resulted in a modification of previous methods for the determination of total ascorbic acid in plasma and whole blood.

2. Studies on the passage of L-ascorbic acid and dehydroascorbic acid from plasma to erythrocytes *in vitro* were made on adult and fetal blood from guinea pigs and human subjects in order to clarify the results of the *in vivo* experiments on placental transfer. The results showed that after an addition of L-ascorbic acid to adult and fetal blood of both species, nearly all of the added vitamin remained in the plasma, and only a very

slight increase of the total ascorbic acid content was found in the erythrocytes. After additions of dehydroascorbic acid, on the other hand, to adult and fetal human blood, a marked increase of total ascorbic acid was found in the erythrocytes, and only a slight increase was observed in the plasma. The permeation of dehydroascorbic acid into adult and fetal human erythrocytes occurred at equal rates. Experiments on adult and fetal blood from guinea pigs showed that dehydroascorbic acid penetrated into the erythrocytes of fetal guinea pigs at a rate corresponding to that found in human blood, whereas the passage into adult guinea pig erythrocytes was considerably slower.

3. The effect of temperature on the passage of dehydroascorbic acid into human erythrocytes was studied at 0°C, 23°C and 37°C. The temperature coefficient, Q_{10}, as calculated from these studies, was about 1.2. Further experiments on the mechanism of transfer of dehydroascorbic acid across the erythrocyte membrane showed that the presence of Hg^{++} ions in a concentration of $2 \cdot 10^{-5}$ M did not alter the rate of transfer.

4. *In vivo* experiments on guinea pigs, in which L-ascorbic acid or dehydroascorbic acid was infused into the maternal circulation, showed upon analysis of successive blood samples of mother and fetus, that infused L-ascorbic acid only slightly increased the values of total ascorbic acid in the fetal blood, whereas an infusion of dehydroascorbic acid produced a marked increase of total ascorbic acid, especially in the fetal erythrocytes. In the experiments in which dehydroascorbic acid was infused, a clear increase of total ascorbic acid was found in the amniotic fluid. This increase was not found when L-ascorbic acid was infused.

5. In experiments in which the effect of hypothermia on the placental transfer of dehydroascorbic acid was studied by lowering the temperature of one fetus to about 27°C during the infusion, only a slight difference in the rate of change in concentration of total ascorbic acid in the blood of the cold and the warm fetus was found. The oxygen saturation of the umbilical vein, however, increased in the cold fetus from 50 % to about

75 %. The possible changes in the placental circulation at lowered temperature have been discussed.

6. In studies on samples of maternal and cord blood from human subjects, it was found that in all cases studied the fetal blood contained more total ascorbic acid (mean 2.01 mg %) than the maternal blood (mean 1.24 mg %). Dehydroascorbic acid was distributed more equally (mean in fetal plasma 0.40 mg % and in maternal plasma 0.38 mg %) on both sides of the placenta, whereas the fetal plasma contained more L-ascorbic acid (mean 1.06 mg %) than the maternal plasma (mean 0.40 mg %). Studies relating the total ascorbic acid content of the amniotic fluid to that of maternal and fetal plasma showed that when the maternal plasma total ascorbic acid exceeded the mean normal value of 0.60 mg %, an accumulation of total ascorbic acid was found in the amniotic fluid, whereas when the fetal plasma showed values below the mean normal value of 1.20 mg % the amniotic fluid contained an even lower total ascorbic acid concentration.

7. Experimental studies on human subjects in which L-ascorbic acid was injected into the mother, and samples of blood taken from the umbilical cord during legal abortion, showed a slight but distinct increase of total ascorbic acid in the fetal plasma. The mechanism of transfer of vitamin C across the placental barrier, and its possible functions during intrauterine life, have been discussed.

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Experimental data of t

Exp.	Date	Before infusion: 0 — Time of blood sampling	Maternal Plasma mg % TAA	Maternal Plasma mg % DHA	Maternal Cell mg % TAA	Fetal Plasma mg % TAA	Fetal Plasma mg % DHA	Fetal Cell mg % TAA	Amniotic fluid mg % TAA	Infusion: Time of infusion	Infusion: Amount infused, mg.	I — Time of blood sampling	Maternal Plasma mg % TAA	Maternal Plasma mg % DHA	Maternal Cell mg % TAA	Fetal Plasma mg % TAA	Fetal Plasma mg % DHA	Fetal Cell mg % TAA	Amniotic fluid mg % TAA
T 1	17.5 -56	10.31 10.35	0.95			0.90				10.43 10.53	2 AA	10.51 10.54	2.10			1.05			
T2	18.5 -56	10.54 10.55	1.10			0.65				10.55 11.05	4 AA	11.03 11.05	4.00			1.80			
T3	25.5 -56	10.45 10.47	0.90			0.55				10.48 10.58	4 AA	10.54 10.57	2.90			0.67			
T4	30.5 -56	10.58 11.00	0.85			0.55				11.00 11.10	4 AA	11.01 11.05	5.00			0.60			
T5	18.6 -56	11.37 11.46	1.10			0.60				11.46 11.56	6 AA	11.54 11.57	3.00			1.10			
T6	9.11 -56	13.30 13.39	0.60			0.50				13.39 13.49	4 AA	13.44 13.47	3.90			0.30			
T7	12.11 -56	11.47 11.54	1.30			0.70				11.55 12.15	5 AA	12.03 12.06	7.80			0.85			
T12	29.1. -57	9.28 9.33	2.10		1.22	0.70		1.30		9.34 9.54	7 AA	9.45 9.47	7.30		2.90	1.00		1.10	
A1	6.6 -57	10.25 10.36	1.50		0.80	1.30		0.80		10.36 10.46	50 AA	10.41 10.46	48.00		18.00	9.40		2.20	
A4	19.6 -57	9.44 9.57	1.20	1.20	1.42	1.40	0.90	1.40	1.10	9.58 10.08	25 AA	10.01 10.04	10.20	5.20	3.30	2.95	0.60	1.86	1.00
A5	26.6 -57	10.14 10.32	1.70	1.10	1.27	3.20	0.50	1.80	3.15	10.36 10.46	25 AA	10.41 10.44	10.60	3.50	2.50	5.80	1.10	2.00	3.90
A7	4.7 -57	10.14 10.32	1.50	1.30	0.62	2.55	0.85	1.46	2.95	10.32 10.42	20 AA	10.36 10.40	8.90	2.70	1.90	2.65	0.55	1.96	2.25
A9	12.7 -57	9.55 10.08	0.45	0.40	1.10	0.60	0.10	1.20	1.05	10.08 10.28	20 AA	10.14 10.16	2.40	1.40	2.40	1.45	1.00	1.80	1.05
A12	23.7 -57	9.50 10.07	0.60	0.60	0.60	0.90	0.50	1.40		10.07 10.27	20 AA	10.16 10.17	4.00	1.20	2.45	1.00	0.55	2.00	
T8	7.1 -57	10.55 11.00	2.70			1.10			0.70	11.00 11.20	10 DHA	11.08 11.11	3.10			1.00			1.10
T9	8.1 -57	10.35 10.37	0.80			0.30				10.40 11.00	4 DHA	10.50 10.51	0.90			0.30			
T10	9.1 -57	10.18 10.37	2.00			2.10			0.75	10.40 11.00	4 DHA	10.49 10.50	2.40			2.00			
T11	28.1 -57	10.04 10.11	1.25		2.66	0.60		1.40		10.12 10.32	5 DHA	10.20 10.21	2.80		4.50	0.95		1.25	

TABLE III

ental data of the infusion experiments performed on guinea pigs.

			After beginning of infusion																							
			II								III								IV							
		Amniotic fluid	Time of blood sampling	Maternal			Fetal			Amniotic fluid	Time of blood sampling	Maternal			Fetal			Amniotic fluid	Time of blood sampling	Maternal			Fetal			Amniotic fluid
	Cell			Plasma		Cell	Plasma		Cell			Plasma		Cell	Plasma		Cell			Plasma		Cell	Plasma		Cell	
mg % A	mg % TAA	mg % TAA		mg % TAA	mg % DHA	mg % TAA	mg % TAA	mg % DHA	mg % TAA	mg % TAA		mg % TAA	mg % DHA	mg % TAA	mg % TAA	mg % DHA	mg % TAA	mg % TAA		mg % TAA	mg % DHA	mg % TAA	mg % TAA	mg % DHA	mg % TAA	mg % TAA
			11.05 11.09	1.45			1.00				11.25 11.28	1.25			1.05											
			11.12 11.15	3.45			1.55																			
			11.05 11.07	1.95			0.95				11.14 11.15	1.75			1.00											
			11.09 11.12	3.05			0.95				11.19 11.24	2.10			1.15											
			12.06 12.08	1.70			0.95				12.16 12.18	1.80			0.90											
			13.51 13.54	3.00			0.40				13.59 14.01	1.70			0.40				14.24 14.25	0.75			0.45			
			12.09	6.80							12.15 12.16	3.80			1.05											
	1.10		9.54 9.55	3.75		1.00	0.90		0.90																	
	2.20																									
	1.86	1.00	10.10 10.14	14.70	7.30	4.20	3.90	3.70	1.20	1.10	10.22 10.26	11.40	5.50	4.20	4.70	1.90	1.90		10.33 10.34				4.40	3.60	1.30	1.00
	2.00	3.90	10.46 10.54	13.20	5.20	3.30	4.50		2.00	3.90	10.56 10.59	10.20	3.00	3.10	4.10	1.30	2.70									
	1.96	2.25	10.41 10.44	9.90	3.70	1.70	3.15	1.15	1.90		11.12 11.15	8.40	2.70	1.60	3.85	1.01	1.20	2.70								
	1.80	1.05	10.24 10.26	6.85	3.00	2.55	3.15	1.35	1.20		10.37 10.40	4.65	2.45	3.30	2.90	1.10	0.60	1.35	10.50 10.52	4.55	2.05	2.55	2.75	1.25	0.80	
	2.00		10.26 10.28	7.10	1.65	2.90	1.30	0.55	1.60		10.42 10.44	4.90	1.75	1.80	1.60	0.90	1.20		10.57 11.00	4.40	1.50	1.65	1.30	0.70	0.90	
		1.10	11.20 11.24	3.70			1.10				11.48 11.50	3.10			1.00											
			11.01 11.02	1.20			0.35				11.20 11.21	1.20			0.35											
	1.25		11.35 11.38	1.90		2.55	0.70		1.50																	

T 1	17.5 -56	10.31 10.35	0.95			0.90				10.43 10.53	2 AA	10.51 10.54	2.10			1.05			
T2	18.5 -56	10.54 10.55	1.10			0.65				10.55 11.05	4 AA	11.03 11.05	4.00			1.80			
T3	25.5 -56	10.45 10.47	0.90			0.55				10.48 10.58	4 AA	10.54 10.57	2.90			0.67			
T4	30.5 -56	10.58 11.00	0.85			0.55				11.00 11.10	4 AA	11.01 11.05	5.00			0.60			
T5	18.6 -56	11.37 11.46	1.10			0.60				11.46 11.56	6 AA	11.54 11.57	3.00			1.10			
T6	9.11 -56	13.30 13.39	0.60			0.50				13.39 13.49	4 AA	13.44 13.47	3.90			0.30			
T7	12.11 -56	11.47 11.54	1.30			0.70				11.55 12.15	5 AA	12.03 12.06	7.80			0.85			
T12	29.1. -57	9.28 9.33	2.10		1.22	0.70		1.30		9.34 9.54	7 AA	9.45 9.47	7.30		2.90	1.00		1.10	
A1	6.6 -57	10.25 10.36	1.50		0.80	1.30		0.80		10.36 10.46	50 AA	10.41 10.46	48.00		18.00	9.40		2.20	
A4	19.6 -57	9.44 9.57	1.20	1.20	1.42	1.40	0.90	1.40	1.10	9.58 10.08	25 AA	10.01 10.04	10.20	5.20	3.30	2.95	0.60	1.86	1.00
A5	26.6 -57	10.14 10.32	1.70	1.10	1.27	3.20	0.50	1.80	3.15	10.36 10.46	25 AA	10.41 10.44	10.60	3.50	2.50	5.80	1.10	2.00	3.90
A7	4.7 -57	10.14 10.32	1.50	1.30	0.62	2.55	0.85	1.46	2.95	10.32 10.42	20 AA	10.36 10.40	8.90	2.70	1.90	2.65	0.55	1.96	2.25
A9	12.7 -57	9.55 10.08	0.45	0.40	1.10	0.60	0.10	1.20	1.05	10.08 10.28	20 AA	10.14 10.16	2.40	1.40	2.40	1.45	1.00	1.80	1.05
A12	23.7 -57	9.50 10.07	0.60	0.60	0.60	0.90	0.50	1.40		10.07 10.27	20 AA	10.16 10.17	4.00	1.20	2.45	1.00	0.55	2.00	
T8	7.1 -57	10.55 11.00	2.70			1.10			0.70	11.00 11.20	10 DHA	11.08 11.11	3.10			1.00			1.10
T9	8.1 -57	10.35 10.37	0.80			0.30				10.40 11.00	4 DHA	10.50 10.51	0.90			0.30			
T10	9.1 -57	10.18 10.37	2.00			2.10			0.75	10.40 11.00	4 DHA	10.49 10.50	2.40			2.00			
T11	28.1 -57	10.04 10.11	1.25		2.66	0.60		1.40		10.12 10.32	5 DHA	10.20 10.21	2.80		4.50	0.95		1.25	
A2	12.6 -57	10.20 10.30	1.30		1.30	0.75		0.86	0.90	10.31 10.41	24 DHA	10.39 10.43	8.80		13.20	3.00			2.20
A3	14.6 -57	9.50 10.04	0.45		1.22	0.60		0.80	0.80	10.07 10.17	21 DHA	10.13 10.18	6.10		4.76	1.45		7.00	1.30
A6	28.6 -57	9.52 10.07	0.80	0.70	0.69	0.70	0.60	0.80	1.20	10.09 10.19	21,5 DHA	10.14 10.17	3.65	3.45	3.55	1.45	1.15	6.05	1.30
A10	16.7 -57	9.47 10.01	0.55	0.50	1.55	0.80	0.50	0.80		10.02 10.12	21 DHA	10.09 10.11	16.00	15.30	13.40	1.60	0.90	5.60	
A14	8.8 -57	9.54 10.14	0.90	0.90	0.25	0.70	0.70	0.70		10.15 10.25	19 DHA	10.24 10.26	4.00	3.90	4.70	1.30	0.80	7.31	

		11.05 11.09	1.45			1.00				11.25 11.28	1.25			1.05											
		11.12 11.15	3.45			1.55																			
		11.05 11.07	1.95			0.95				11.14 11.15	1.75			1.00											
		11.09 11.12	3.05			0.95				11.19 11.24	2.10			1.15											
		12.06 12.08	1.70			0.95				12.16 12.18	1.80			0.90											
		13.51 13.54	3.00			0.40				13.59 14.01	1.70			0.40				14.24 14.25	0.75			0.45			
		12.09	6.00							12.15 12.16	3.80			1.05											
1.10		9.54 9.55	3.75		1.00	0.90		0.90																	
2.20																									
1.86	1.00	10.10 10.14	14.70	7.30	4.20	3.90	3.70	1.20	1.10	10.22 10.26	11.40	5.50	4.20	4.70	1.90	1.90		10.33 10.34				4.40	3.60	1.30	1.00
2.00	3.90	10.46 10.54	13.20	5.20	3.30	4.50		2.00	3.90	10.56 10.59	10.20	3.00	3.10	4.10	1.30	2.70									
1.96	2.25	10.41 10.44	9.90	3.70	1.70	3.15	1.15	1.90		11.12 11.15	8.40	2.70	1.60	3.85	1.01	1.20	2.70								
1.80	1.05	10.24 10.26	6.85	3.00	2.55	3.15	1.35	1.20		10.37 10.40	4.65	2.45	3.30	2.90	1.10	0.60	1.35	10.50 10.52	4.55	2.05	2.55	2.75	1.25	0.80	
2.00		10.26 10.28	7.10	1.65	2.90	1.30	0.55	1.60		10.42 10.44	4.90	1.75	1.80	1.60	0.90	1.20		10.57 11.00	4.40	1.50	1.65	1.30	0.70	0.90	
	1.10	11.20 11.24	3.70			1.10				11.48 11.50	3.10			1.00											
		11.01 11.02	1.20			0.35				11.20 11.21	1.20			0.35											
.25		11.35 11.38	1.90		2.55	0.70		1.50																	
	2.20	10.47 10.53	6.70		10.50	2.80																			
.00	1.30	10.22 10.29	4.50		3.16	2.05		9.00																	
.05	1.30	10.28 10.34	3.90	3.60	3.40	1.80	1.60	6.90	1.40	10.59 11.05	2.55	2.15	3.50	1.70	1.30	5.50	2.15								
.60		10.19 10.20	4.80	4.30	5.70	2.15	1.15	8.80		10.29 10.30	3.55	3.00	4.10	2.30	1.40	8.50		10.39 10.40	3.10	2.80	3.80	2.85	1.85	7.95	
.31		10.32 10.36	3.00	2.90	3.80	1.50	1.05	7.50		10.45 10.48	3.30	3.00	3.85	1.70	1.20	6.70		10.58 11.01	2.45	2.25	3.65				